2857-Pos Board B549
Localized Nitric Oxide Signaling Mediates Cardiac Mechano-Chemotransduction
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Cardiac myocytes contract against a mechanical afterload during each heartbeat. We have developed a novel Cell-in-Gel system to impose 3D mechanical stresses on single cardiac myocytes during contraction. Using this new technique, we identified key molecules involved in transducing mechanical stress to alter Ca^{2+} dynamics. Increasing mechanical load causes enhanced contractility and elevated systolic Ca^{2+} transient that is mediated by nitric oxide synthase (NOS). Increased load also causes a marked increase of diastolic spontaneous Ca^{2+} sparks, and their suppression is only effected by inhibition of nNOS but not eNOS. The differential effects on Ca^{2+} sparks may stem from the two-fold closer physical proximity of nNOS vs. eNOS to ryanodine receptors. In addition to NOS, NOX2 and CaMKII are also involved in the mechano-chemotransduction pathways, which together fine-tune cardiac contraction under mechanical load.

2858-Pos Board B550
Cardiomyopathy Ctnt Mutation in Patient Derived Cardiomyocytes from Induced Pluripotent Stem Cells Affects Sarcomere Structure and Function
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Cardiomyocytes derived from human induced pluripotent stem cells (hIPS-CM) are useful to understand basic structural and functional characteristics of normal and diseased human heart cells. We investigated mechanical properties of hIPS-CM derived from unaffected and affected members of a family harboring a dilated cardiomyopathy (DCM) mutation in cardiac troponin T (cTnT), the tropomyosin binding unit of troponin. Patients with a cTnT point mutation (R173W) develop DCM, which commonly leads to diastolic and systolic dysfunction and progressive heart failure. To study the shortening and shortening velocity of normal and cTnT mutant hIPS-CM cells, cells were plated on substrates with a relatively soft stiffness (160 kPa) molded from polydimethylsiloxane and measured using a line scan method. Data were collected using a Zeiss 710 confocal microscope. Visual observation of cells after 5 days maturity on the PDMS substrates indicate a smaller percent of mutant (8%) compared to normal (87%) hIPS-CM were spontaneously beating. Immunohistochemistry showed that myofibril structure was better developed in normal compared to cTnT mutation cells. Day 5 line scans revealed normal hIPS-CM cells shorten more (0.47 μm) than mutant cTnT cells (0.32 μm); shortening velocity was faster in normal (1.42 μm/s) compared to mutant cTnT cells (0.67 μm/s). Acute treatment (1 dose with a 10 min activation dwell time) with omecamtiv mecarbil (200nM), a cardiac myosin activator, increased the shortening and shortening velocity of normal (0.78 μm at 1.85 μm/s) but not cTnT mutant (0.32 μm at 0.70 μm/s) hIPS-CM cells. Although more studies are necessary, these results suggest that cTnT may affect development of sarcomeres and the regulation of contractility. Furthermore, the myosin activator omecamtiv mecarbil may not be sufficient to rescue dysfunction in the cTnT mutation R173W. [Funding: NIH T32HL07692, PO1HL62426]

2859-Pos Board B551
Contractile Structure-Function of Parvalbumin's Ef-Hand Metal Ion Binding Loop in Isolated Adult Cardiac Myocytes
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Parvalbumin (Parv), an EF-hand Ca^{2+} buffer, facilitates rapid relaxation in fast-twitch muscle. Parv gene delivery to the heart has been studied as a therapeutic strategy for diastolic heart failure, in which slow Ca^{2+} reuptake is an important contributor. A limitation of WT-Parv in this context is the significant trade-off between faster relaxation rate and blunting of contraction amplitude, which occurs because WT-Parv sequesters Ca^{2+} too early in the cardiac cycle and prematurely truncates sarcomere shortening in the facilitation of rapid relaxation. Our laboratory recently demonstrated that an E→Q substitution (ParvE101Q) at amino acid 12 of the EF-hand metal-ion binding loop increases Mg^{2+} affinity and decreases Ca^{2+} affinity. Mechanistically, E→Q disrupts bidentate Ca^{2+} binding at this site to reduce Ca^{2+} binding affinity. Functionally, this substitution delays Ca^{2+} buffering compared to WT-Parv in cardiac myocytes, which hastens relaxation without blunting contraction. Unexpectedly, ParvE101Q increases contraction amplitude above that of untreated myocytes and independent of Ca^{2+}, leading to the hypothesis that sarcomere-localized altered Mg^{2+} binding may contribute to the inotropic effect. In this work, to further elucidate the role of EF-hand motif residue 12 in function, ParvE101Q and ParvE101D-experessing cardiac myocytes are compared. Although both Q and D substitutions are thought to bind Ca^{2+} in a monodentate manner, ParvE101D may bind Mg^{2+} more strongly than ParvE101Q. Consistent with this, in preliminary studies, ParvE101D enhanced contraction amplitude to a greater extent than ParvE101Q. We will discuss these findings in the context of the mechanistic role of EF-hand loop residue 12 in conferring Ca^{2+} and Mg^{2+} binding affinities with the goal of optimizing a Ca^{2+} buffering system for heart failure.

2860-Pos Board B552
Phospholamban and Sarcolipin Pentamers Naturally Associate with the Sarcoplasmic Reticulum Calcium Pump
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Phospholamban and sarcolipin interact with the sarcoplasmic reticulum calcium pump (SERCA) and regulate contractility in smooth, cardiac and skeletal muscle. While both proteins can form oligomers, it is thought that only the monomers interact with and inhibit SERCA. To address the role of the phospholamban and sarcolipin pentamers, we have studied their interaction with SERCA using electron cryo-microscopy of two-dimensional co-crystals. In our previous studies, phospholamban oligomers were found interspersed between SERCA dimers and we constructed a three-dimensional model of the complex. We also addressed the molecular characteristics of phospholamban that contribute to its interaction with SERCA and we examined the effects of phosphorylation and mutation of phospholamban on the structure of the complex with SERCA. In our recent work, we compared two crystal forms of SERCA in the absence and presence of phospholamban by electron cryo-microscopy - namely, small helical crystals and large two-dimensional crystals. The SERCA dimer ribbons that are found in both crystal forms consist of a rigid assembly of calcium-free SERCA molecules. While the lattice formed by the SERCA dimer ribbons is different in the helical and two-dimensional crystals, we show that a phospholamban oligomer interacts with SERCA in a similar manner in both crystal types. With this information, we next undertook a structural investigation of SERCA and sarcolipin in the two-dimensional crystals. A projection map was determined for SERCA in the presence of sarcolipin to a resolution of 8.5 Å and was consistent with a pentameric state for sarcolipin. While both phospholamban and sarcolipin interacted with transmembrane segment M3 of SERCA, the interaction of the sarcolipin pentamer was mediated by an additional density consistent with a SLN monomer. We conclude that pentameric forms of both phospholamban and sarcolipin naturally associate with SERCA.

2861-Pos Board B553
Phospholamban C-Terminal Truncations Including Heart Failure Mutation L39Stop Decrease Membrane Localization and Oligomerization and Alter the Structure of the PLB-Serca Complex
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A naturally occurring missense Leu-39stop (L39X) mutation in phospholamban (PLB) results in truncation of the C-terminal transmembrane domain, leading to cardiomyopathy and premature death. In this study, we fused PLB and SERCA to fluorescent protein tags to determine the structural and thermodynamic consequences of progressive truncations of the C-terminal residues of PLB in the membranes of living cells. We found that deletion of only a few C-terminal residues resulted in significant loss of PLB membrane anchoring and mislocalization to the cytoplasm and nucleus. Selective permeabilization of the plasma membrane by saponin resulted in diffusion of fluorescently labeled PLB out of the cells, consistent with solubilization of truncated proteins. Western blot analysis showed the expected mobilities for truncation mutants relative to full length PLB-WT, indicating that the observed solubilization of PLB truncation mutants is not due to proteolysis. Moreover, molecular dynamics simulations recapitulated the observed loss of stable bilayer anchoring for truncated PLB. Fluorescence resonance energy transfer (FRET) analysis revealed that C-terminal truncations resulted in progressive loss of PLB-PLB FRET, indicating a decrease in the apparent affinity of PLB oligomerization. We quantified a similar decrease in the SERCA-PLB binding affinity. Despite this decrease in affinity, SERCA-PLB FRET was paradoxically increased by deletion of up to 4 C-terminal residues as a result of a 14 angstrom decrease

in the distance between donor and acceptor fluorophores. Truncation of PLB also decreased its inhibitory potency as quantified by calcium-dependent ATPase activity. We conclude that the C-terminal residues are critical for PLB localization, SERCA-PLB regulatory complex conformation, and PLB regulatory function.

2862-Pos Board B554

Fluorescence Resonance Energy Transfer Reveals that Serca Dimerizes and Forms a Complex with Phospholamban in a 2:1 Stoichiometry

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The sarco/endoplasmic reticulum calcium ATPase (SERCA) has been proposed to form functional dimers *in vitro*. In order to investigate whether SERCA forms homo-dimers in live cells, we fused canine SERCA2a to cerulean (Cer) or yellow fluorescent protein (YFP), and quantified SERCA-SERCA interactions by fluorescence resonance energy transfer (FRET). SERCA-SERCA FRET efficiency was dependent on the labeling position of the fluorescent protein tags, with the highest FRET efficiency achieved when the respective fluorescent proteins were fused to SERCA N-termini. FRET was reduced by competition with unlabeled SERCA, suggesting that the observed FRET was due to specific protein-protein interactions. Progressive photobleaching of YFP showed that Cer intensity increased linearly with decreasing YFP intensity, suggesting that the stoichiometry of the SERCA complex is a dimer. In contrast, a control experiment with phospholamban (PLB) oligomer showed a non-linear YFP/Cer relationship, consistent with its well-known pentameric stoichiometry. We also investigated whether SERCA dimers could interact with PLB, the regulatory binding partner of SERCA. Interestingly, while average maximal FRET was 28% between SERCA and PLB, fluorescence lifetime measurements revealed two different lifetimes, consistent with two different populations of FRET donors. One population showed very low FRET, while the other population exhibited high FRET- approximately double the measured average maximal FRET efficiency. The data are consistent with a single PLB bound to each SERCA homo-dimer; in this regulatory complex one SERCA protomer is in close proximity to PLB, while the other is too far away to participate in FRET with PLB.

2863-Pos Board B555

Clinical Trials of Gene Therapy for Heart Failure: Quantitation of the Cardiac Calcium Pump by Immunochemistry

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We have used quantitative immunoblot, enzyme-linked immunosorbant assay (ELISA), and capillary western to measure absolute cardiac sarcoplasmic reticulum Ca^{2+} ATPase (SERCA2a isoform) content in order to determine specific activity. Celladon, Inc., is developing a gene therapy treatment for heart failure (HF) where SERCA2a is the target protein, and they need an accurate measure of SERCA2a to calculate specific activity of enzyme produced by the gene therapy construct. Patients with HF produce too little SERCA2a or SERCA2a that has too low of activity to maintain normal calcium cycling that is responsible for cardiac contraction. SERCA2a produced by MYDICAR, an adeno- associated viral (AAV) vector, must have comparable activity to endogenous SERCA2a to be useful as a gene therapy product. Immunoblot showed linear SERCA2a detection. We conclude that immunoblot measures absolute SERCA2a content, useful in measuring specific activity. This work was funded by Celladon and by NIH grants to DDT (R01 GM27906, P30 AR0507220, T32 AR007612).

2864-Pos Board B556

Sarcoplasmic Reticulum-Independent Contractile Function in Serca2 Ablated Hearts

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Effective contraction and relaxation of the heart requires the robust transport of Ca^{2+} to and from the sarcoplasmic reticulum (SR). The cardiac SR Ca-ATPase, SERCA2a, is responsible for sequestering Ca^{2+} to the SR during the contractile cycle, and has diminished expression and function in failing hearts. We have used a model of inducible cardiac-specific Serca2 gene disruption, the Serca2fl/fl mouse, to probe the relationship between decreased SERCA2a expression and cardiac dysfunction. 4 weeks after gene disruption, SERCA2a protein content in KO hearts is less than 5% of control. Despite severely impaired ex vivo function of isolated hearts and cardiomyocytes at this time, in vivo heart function is mildly impaired and KO mice survive until 7-10 weeks post-knockout, indicating that loss of SERCA2a is temporarily compensated. This finding of preserved cardiac function in vivo in the context of severe SERCA2a depletion is surprising and warrants a detailed dissection of potential compensatory mechanisms. Published studies have found that sarcolemmal Ca^{2+} transport mechanisms such as L-type Ca^{2+} current, Na-Ca exchange, and plasma membrane Ca-ATPase activity are increased following this loss of SERCA2a protein. These observations prompt the hypothesis that sarcolemmal Ca^{2+} transport remodeling may partially compensate for diminished SR Ca^{2+} handling in KO hearts. We have begun to investigate this hypothesis by perfusing isolated Serca2 KO hearts with caffeine to evaluate SR-independent contractile function. During caffeine perfusion, left ventricular (LV) end-diastolic pressures were lower in KO hearts than controls consistent with the reduced SR Ca^{2+} stores in KO myocytes. Interestingly, during prolonged caffeine perfusion, systolic performance was similar in control and KO hearts. This is functional evidence that transsarcolemmal transient adaptations in KO hearts are not of sufficient magnitude to differentiate KO from WT when SR function is disabled by caffeine.

2865-Pos Board B557

Simultaneous Imaging of Local Calcium and Single Sarcomere Length in Rat Neonatal Cardiomyocytes via Expression of Cameleon-Nano in Z-Discs

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In cardiac muscle, contraction is regulated by micromolar concentrations of Ca^{2+} released from the sarcoplasmic reticulum (SR) (i.e., Ca^{2+}-induced Ca^{2+} release), resulting in the binding of Ca^{2+} to troponin C and subsequent formation of cross-bridges. In order to enhance our understanding of cardiac excitation-contraction coupling, we in the present study developed a novel experimental system for simultaneous measurement of local intracellular Ca^{2+} ($[Ca^{2+}]_i$) and single sarcomere length via expression of yellow cameleon-Nano (i.e., FRET-based ultrasensitive Ca^{2+} indicator) fused to the C-terminus of α-actinin in Z-discs in primary-cultured rat neonatal cardiomyocytes. In a given sarcomere, the fluorescence emission ratio (YFP/CFP) of the expressed fusion protein varied in response to a change in local $[Ca^{2+}]_i$. Under dual-view microscopy, we simultaneously measured local $[Ca^{2+}]_i$ and sarcomere length (the latter defined as the distance between the YFP fluorescence profiles), and found that sarcomere length varied in response to a change in YFP/CFP in various local regions of the myocyte. These results demonstrate that the present experimental system is useful for elucidating cardiac excitation-contraction coupling at the single sarcomere level.

2866-Pos Board B558

Real-Time Intracellular Calcium Imaging in the Heart

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In the present study, we developed an experimental model for real-time imaging of intracellular Ca^{2+} in ventricular myocytes in the heart. First, the heart isolated from the adult mouse was mounted on the stage of a microscope with a confocal scanning unit, combined with an objective lens [20× (40×), numerical aperture 1.0 (0.8)]. Laser-excited fluorescence was detected by the EMCCD camera, and the signals were analyzed by using the ImageJ software. Ca^{2+} waves were clearly observed at the cellular level in the isolated heart. Interestingly, randomly occurring Ca^{2+} waves and/or transients became synchronized by electric stimulation (~5 Hz). Next, we conducted the isolated heart experiments at various temperatures. We found that 1) higher temperatures (~37°C) reduced the efficiency of loading Ca^{2+} indicator into the cardiomyocytes and 2) the fluorescence intensity of Ca^{2+} waves were less pronounced at ~37°C than at ~25°C. Therefore, temperature control is highly important to allow for intracellular Ca^{2+} imaging in the heart *in vivo*. Intracellular Ca^{2+} imaging in the heart will greatly enhance our understanding of the excitation-contraction coupling in health and disease.